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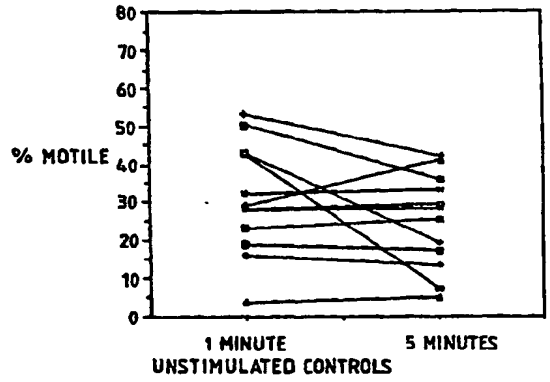
(54) Title: ANGIOTENSIN II FOR IMPROVING FERTILIZATION

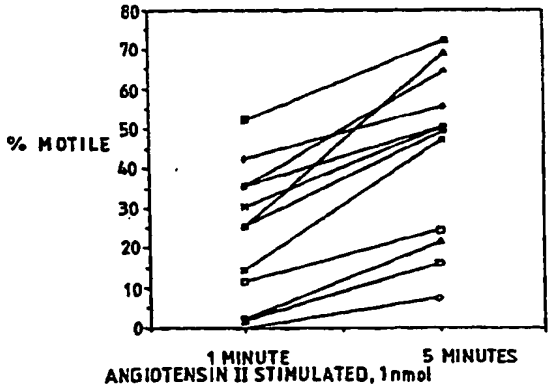
(57) Abstract

The present invention relates to the use of angiotensin II, or an analogue thereof, for promoting the fertilization of mammalian eggs. It also relates to a method of promoting *in vitro* fertilization of mammalian using angiotensin II.



angiotensin II promotes sperm motility

1 nM 1 μM
10 nM



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Angiotensin II For Improving Fertilization

The present invention relates to the use of angiotensin II, or an analogue thereof, for promoting fertilization of mammalian eggs, especially human eggs. In particular, it relates to the use of angiotensin II to improve sperm motility. The invention also relates to a method of promoting in vitro fertilization.

Angiotensin II is an octapeptide, usually regarded as being produced in the blood, firstly by the action of renin, an enzyme secreted by the kidney, on angiotensinogen, resulting in the formation of the decapeptide precursor, angiotensin I, and secondly the action of a dipeptidase "angiotensin converting enzyme"; this enzyme acts on angiotensin I to form angiotensin II. Angiotensin II, in turn, undergoes hydrolysis by an aminopeptidase to yield the heptapeptide angiotensin III (angiotensin 1-7).

The hormone angiotensin II (Ang II) forms part of the renin - angiotensin system which helps to control electrolyte balance and blood pressure within the body. There are several tissues within the body upon which Ang II acts, they include the adrenal gland, uterus, liver, brain and kidney.

Amongst the several established functions of angiotensin II, it is known to be involved in vaso-constriction, which leads to hypertension. Most treatments for high blood pressure will include blockage of angiotensin function in one way or another. Ang II also stimulates the secretion of aldosterone by the adrenal cortex. Aldosterone is a potent hormone which acts primarily on the kidney to promote sodium retention and thus inter alia, heightens the hypertensive effects of angiotensin acting directly on the vasculature.

Ang II is known to act on various sites in the brain, and one of its actions in animals is the regulation of thirst and drinking.

Angiotensin also has trophic effects on the vasculature, promoting growth of the muscles in the arterial

wall. It is also thought to be angiogenic, i.e. it causes vascularisation of newly developing tissue.

Most of the established effects of Ang II have been found to occur via the AT_1 subtype of the Ang II receptor, which is a seven transmembrane domain receptor. This receptor has been cloned and sequenced from a variety of tissues, and has been found to be a 359 amino acid polypeptide with a predicted molecular weight of around 40kD (Bernstein and Alexander, (1992), Endocr. Rev., 13, 381-386). Studies using photo-affinity labelling and crosslinking agents have suggested molecular weights for mature receptor of approximately 65kD and 116 kD, respectively, which may reflect glycosylation of asparagine residues within the extra-cellular domain.

From the recent development of a hybridoma cell line, see Barker, S., et al, J. Mol Endocr., 11, 241-245, (1993), it has been found possible to produce monoclonal antibodies to the AT_1subtype of the Ang II receptor. In consequence, such receptors have been found to exist both on maturing rat and human sperm tails, and on free swimming sperm obtained by vaginal lavage from mated rats, and in human ejaculated sperm.

It has now been found that angiotensin II, or an analogue thereof, increases both the percentage of motile sperm and their linear velocity.

This newly discovered property of angiotensin II enables Ang II to be used to promote fertilization, since the capacity of sperm to fertilise ova is closely related to their motility.

According to one aspect of the invention there is provided the use of angiotensin II or a salt or analogue thereof to promote fertilization of mammalian eggs. Such fertilization may take place in vitro.

According to a second aspect of the invention there is provided the use of angiotensin II or a salt or analogue thereof to increase sperm motility.

According to another aspect of the invention there is provided the use of angiotensin II, or a salt or analogue thereof, for the manufacture of a medicament for use in

promoting fertilization, in particular in vitro fertilization.

Analogues of angiotensin II which may be used for increasing sperm motility, and thereby promoting fertilization, include angiotensin II amide,angiotensin III (angiotensin 1-7) and angiotensin IV (angiotensin 3-8).

Standard procedures for in vitro fertilisation are described in "In vitro fertilisation: a treatment for male infertility", Cohen, J., Edwards, R. et al, 1985, Fertility and Sterility, 44, 422-432. In general, sperm samples are washed in MEM tissue culture medium, centrifuged at approximately 400g, resuspended and recentrifuged. They are then resuspended in a few drops of MEM and 0.5ml of fresh medium is layered on top. After 30min in the incubator at 37°C, the top 30% of the medium, containing essentially motile sperm, is removed and distributed to oocytes (approximately 10,000 sperm per oocyte) in MEM and left for 24hrs for fertilisation to occur.

According to yet another aspect of the invention there is provided a method of promoting in vitro fertilization of mammalian eggs comprising adding angiotensin II or a salt or analogue thereof to incubation medium containing oocytes and sperm.

Angiotensin II, or an analogue thereof, may be added either in the washing stages, or during the final incubation with the oocyte. When added to the incubation medium, angiotensin II, or an analogue thereof, is added at a concentration of preferably 1 nmole/l.

The invention will be further described with reference to the following examples:-

Example 1

Effect of Angiotensin II stimulation on sperm motility

Human sperm samples were obtained from 12 volunteers and patients attending the Newham Hospital assisted fertility clinic. Samples were suspended in modified minimum essential medium with Earle's salts (MEM) and glutamine and viewed in a Makler chamber using an Olympus inverted microscope fitted with an Olympus ARTF-2 video camera. Fields were recorded on video tape and percentage

motility evaluated at 1 min and 5 min. After mixing with MEM alone (unstimulated controls) or with MEM containing 1 nmole/litre angiotensin II amide.

Percentage motility was estimated on playback of the video tapes by freezing the frame to count all of the sperm within a field and then, in forward mode, by counting immotile sperm, i.e. those which within the period of observation did not move to an adjacent square (100 μmetre) on the Makler Chamber grid. In practice, rigid use of this definition was rarely necessary as sperm were either completely immotile or progressed freely.

From Figure 1(a) it can be seen that there was no significant difference in percentage motility after 5 minutes in the unstimulated controls. Within the angiotensin II stimulated group, however, percentage motility after 5 minutes was significantly different from percentage motility after 1 minute.

Example 2

Effect of AT_1 receptor antagonist on sperm motility

A series of six sperm samples, obtained from the same source as Example 1, were mixed either with unmodified MEM, or MEM containing DuP-753 (1 nmol/l)

Percentage motility was estimated in the same way as in Example 1.

From Figure 2 it is evident that, in the presence of DuP-753, (2-n-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole, potassium salt), percentage motility was significantly decreased relative to the untreated controls.

Example 3

Effect of angiotensin II on sperm velocity

Sperm samples were obtained from the same source as Example 1. One series of samples was kept as controls. To a second series Angiotensin II amide (10 nmole/l was added. A third series was treated with monoclonal antibody to the AT_1 receptor before angiotensin was added.

Velocity was measured by timing forwardly progressive sperm traversing the grid on the Makler Chamber and timing them manually.

From Figure 3(a) it can be seen that stimulation with angiotensin II significantly stimulated forward progressive velocity compared with the untreated controls, whilst addition of the monoclonal antibody inhibited the response to angiotensin II.

From Figure 3(b) it can be seen that similar results were obtained for the effect on the percentage of motile sperm.

Example 4

Sperm samples were obtained from the same source as Example 1. One series of samples was kept as controls. A second series was exposed to angiotensin II (1 μmol/l) for five minutes. A third series was exposed to angiotensin II (1 μmol/l) for 5 minutes and then DuP-753 (1μmol/l) was added.

The percentage of motile sperm was measured using the same method as was used in Example 1.

From Figure 4 it can be seen that, when compared with the controls, in most samples angiotensin II enhanced the percentage of sperm which were motile after 5 minutes. This enhancement was generally reduced in the samples to which DuP-753 had been added.

Example 5

Effect of angiotensin on sperm motility

Sperm samples were obtained from the same source as Example 1. One series of samples was kept as controls. To a second series angiotensin II amide (1 nmol/l) was added.

Sperm motility was assayed by using a computer system which measures different aspects of sperm motility, namely curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH).

Figures 5(a) and 5(b) show the percentage of motile sperm in each of two samples, over a 30 minute period in the presence (shaded bars) and absence (clear bars) of angiotensin II amide (1 nmol/l).

Figure 6(a) and 6(b) show the same samples assayed for VCL.

Figure 7(a) and 7(b) show the same samples assayed for ALH.

Figure 8 compares VCL and ALH in control (unstimulated clear symbols) and angiotensin II (AII) stimulated (solid symbols) sperm samples.

From the figures it is clear that angiotensin II amide stimulates both VCL and ALH, both parameters that are associated with increased fertility.

CLAIMS

1. Use of angiotensin II or a salt or analogue thereof to promote fertilization of mammalian eggs.

2. The use as claimed in claim 1 wherein fertilization takes place in vitro.

3. Use of angiotensin II or a salt or analogue thereof to increase sperm motility.

4. Use of angiotensin II or a salt or analogue thereof for the manufacture of a medicament for use in promoting fertilization.

5. The use as claimed in any one of claims 1 to 4 wherein the analogue of angiotensin is angiotensin II amide, angiotensin III (angiotensin 1-7) or angiotensin IV (angiotensin 3-8).

6. The use as claimed in claim 2 wherein angiotensin II is used at a concentration of 1 nmol/l.

7. A method of promoting in vitro fertilization of mammalian eggs comprising adding angiotensin II or a salt or analogue thereof to incubation medium containing oocytes and sperm.

8. A method as claimed in claim 7 wherein the angiotensin is added at a concentration of 1 nmol/l.

FIG. 1(a)

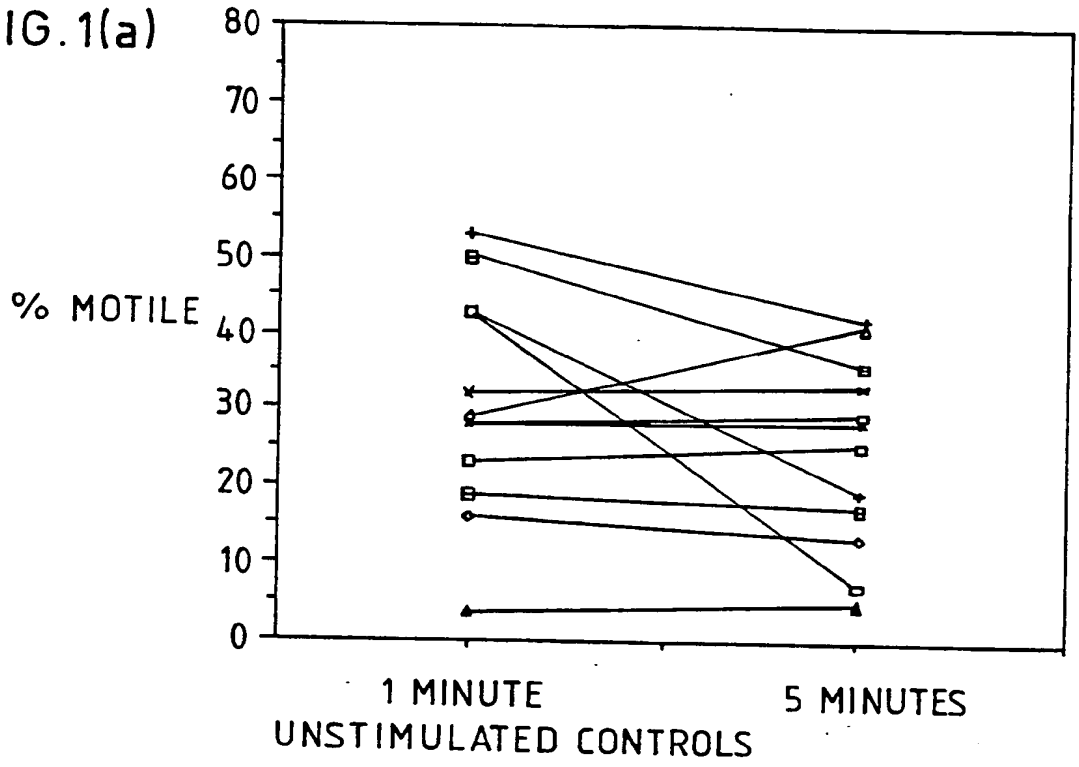


FIG. 1(b)

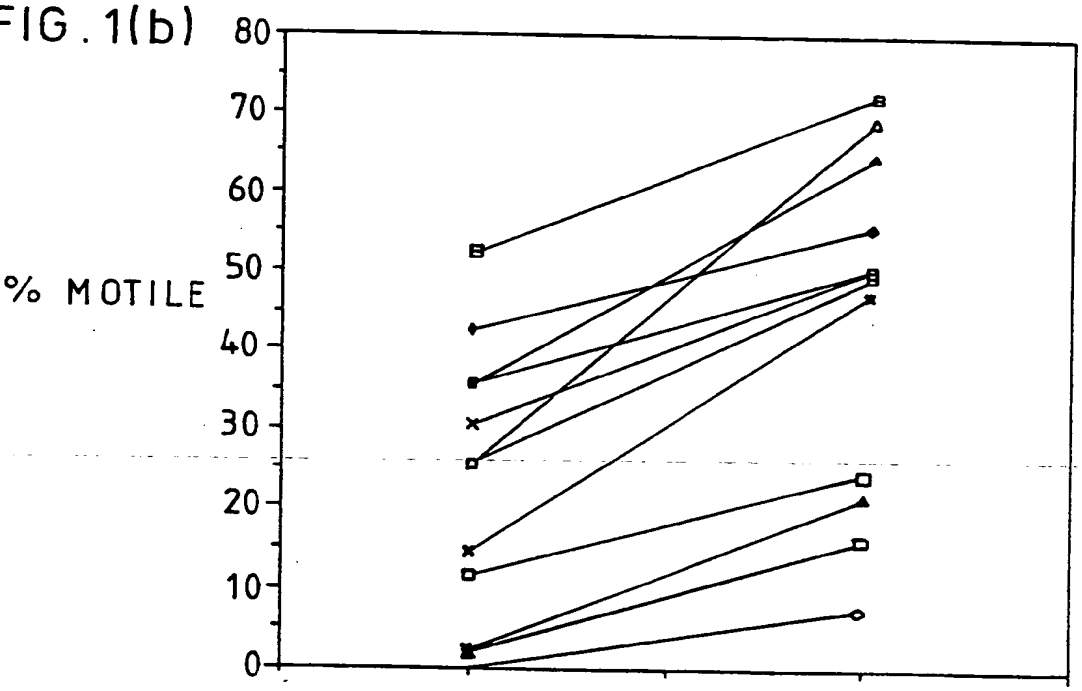


FIG. 2

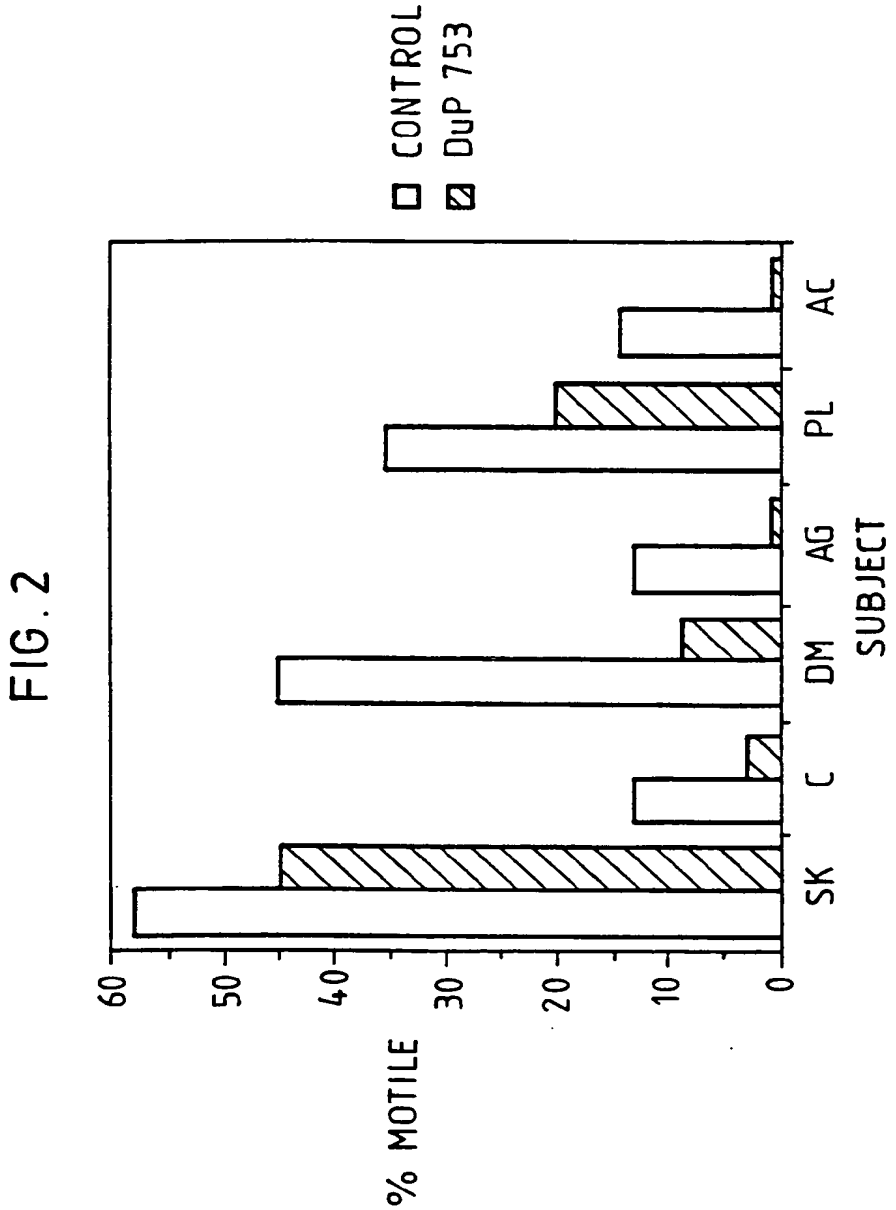


FIG. 3(a)

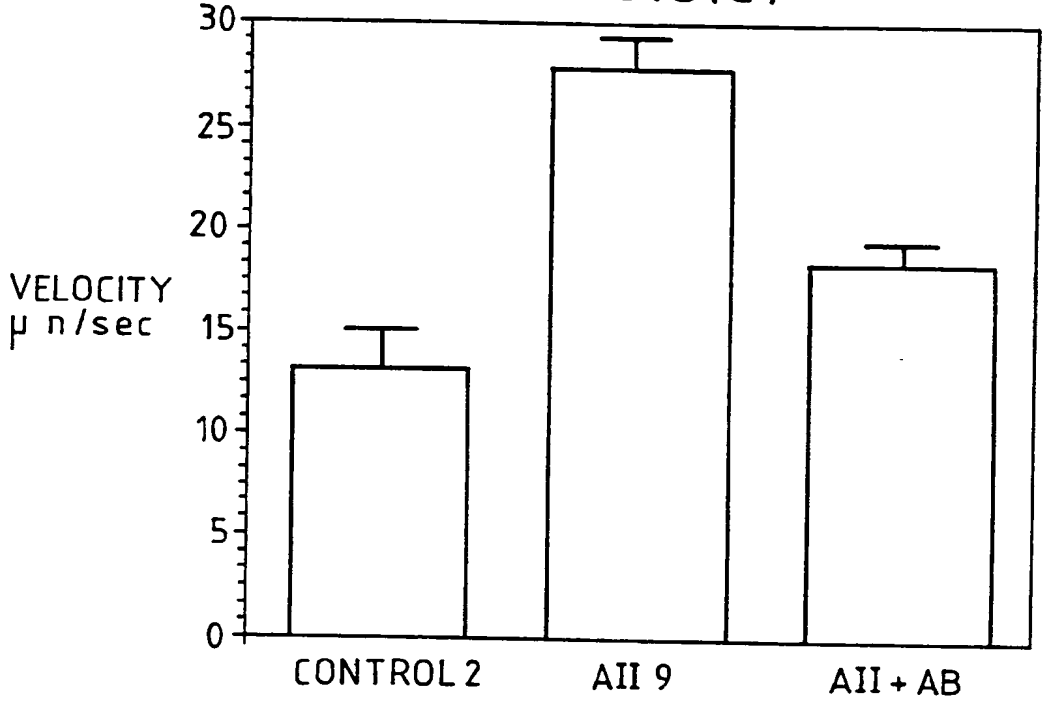


FIG. 3(b)

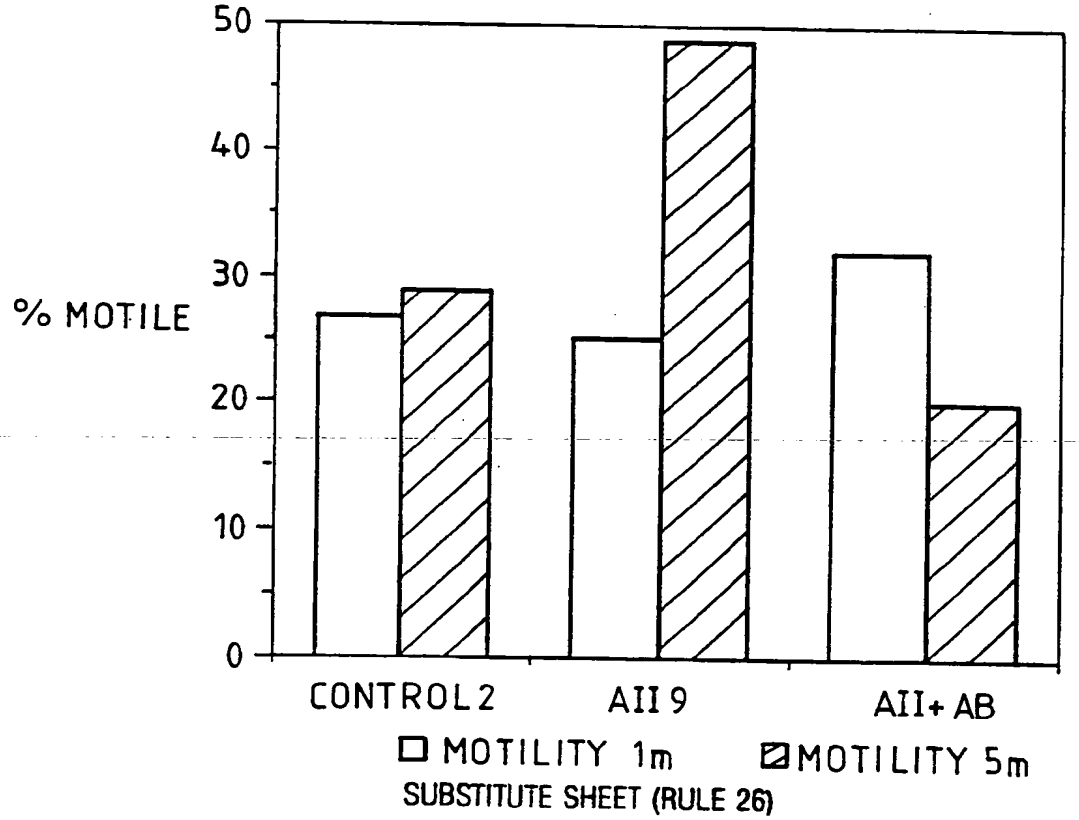


FIG. 4

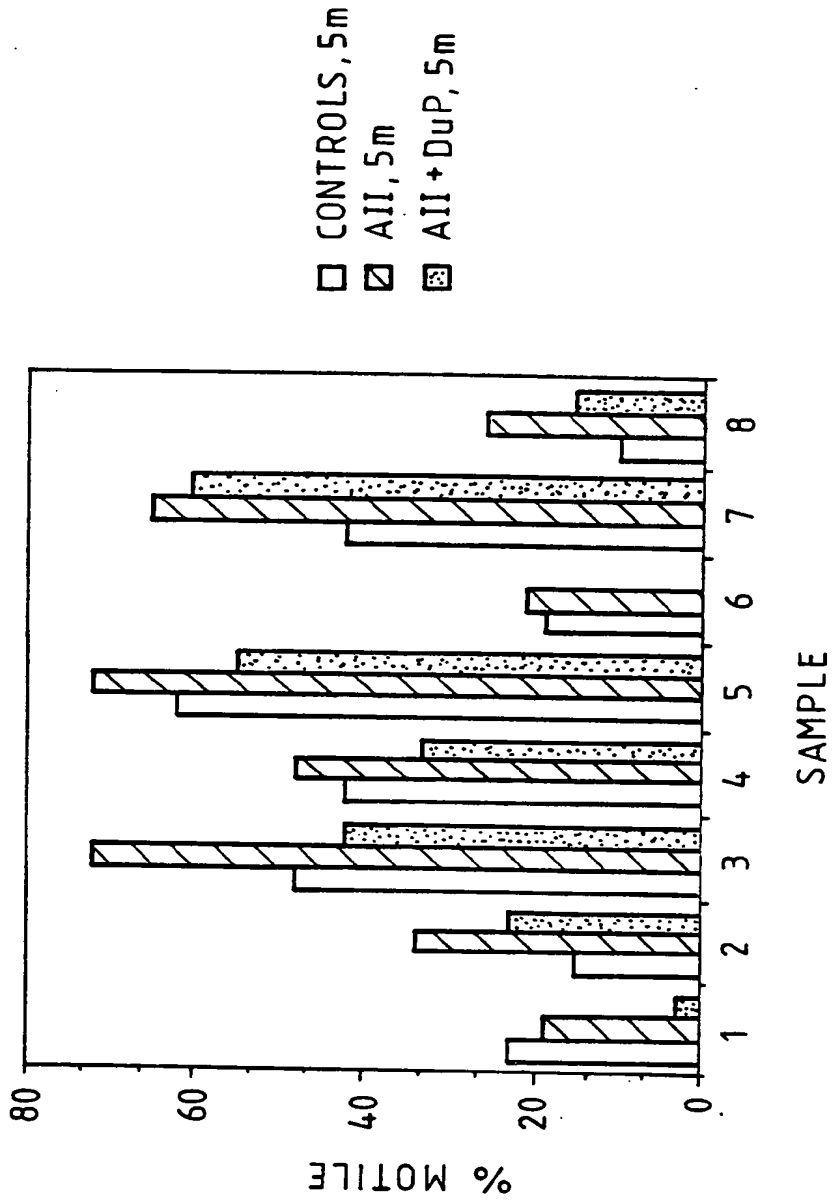


FIG. 5(a)

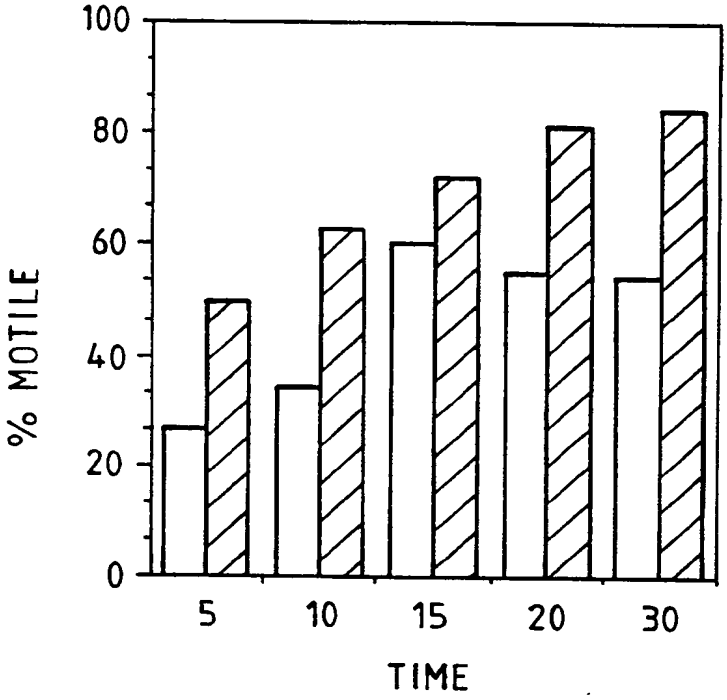


FIG. 5(b)

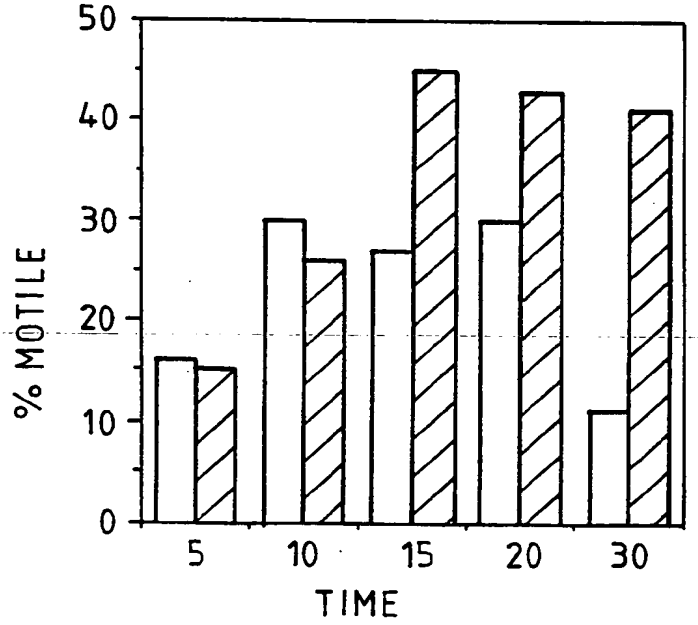


FIG. 6(a)

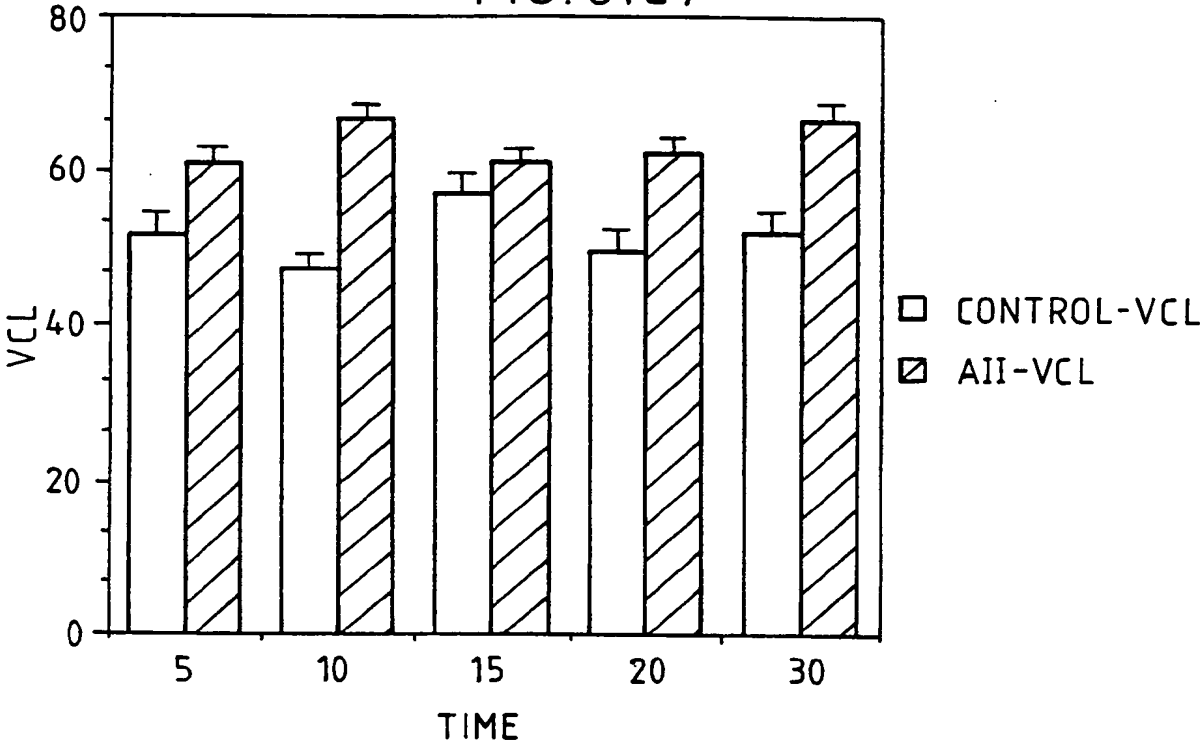


FIG. 6(b)

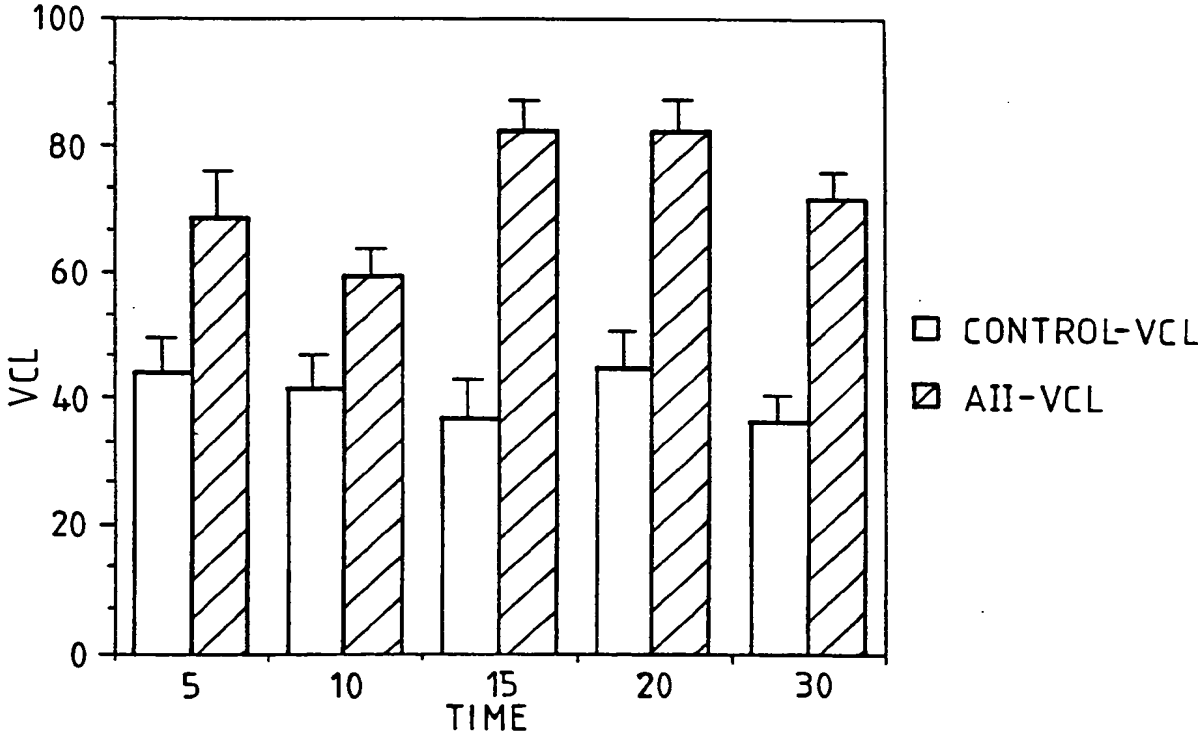


FIG. 7(a)

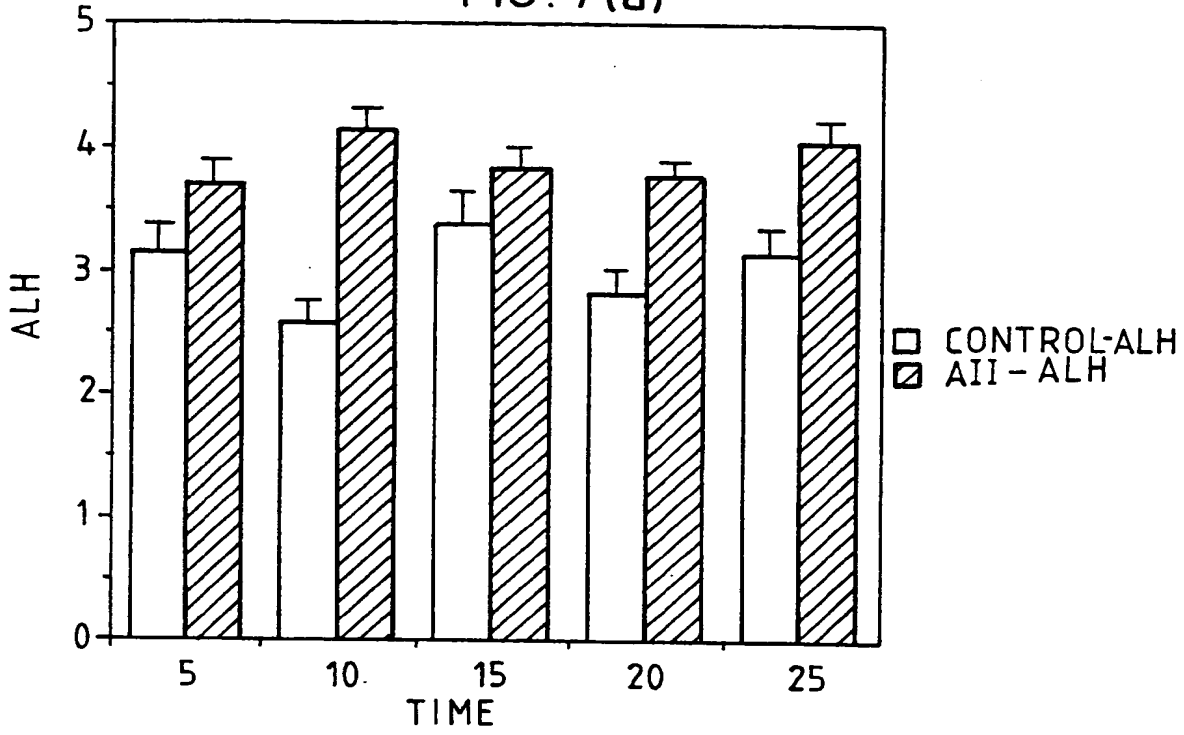


FIG. 7(b)

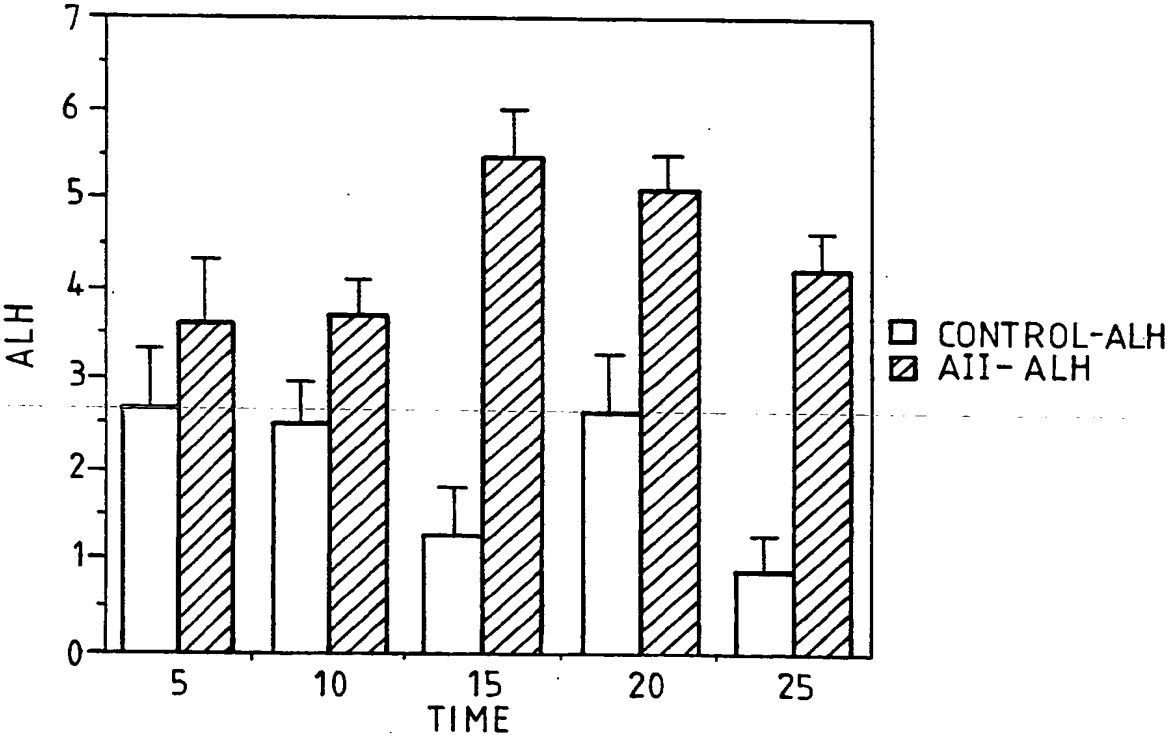
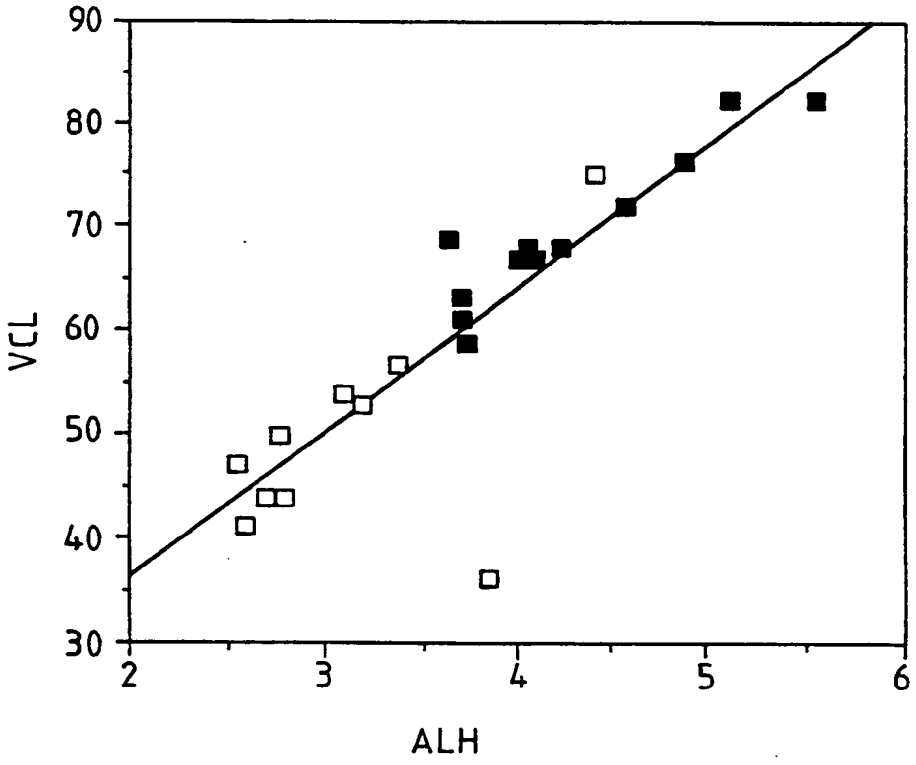


FIG. 8



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 95/01202

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/08 A61D19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO,A,95 09186 (QUEEN MARY AND WESTFIELD COLLEGE) 6 April 1995 see page 5 see example 2 ---	3,5
X	JOURNAL OF PHARMACOBIO-DYNAMICS, vol. 7, no. 2, 1984 TOKYO, pages 87-93, KANEKO S. ET AL 'Effects of Angiotensin on the motility of human sperm' see the whole document ---	1-8
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 September 1995	03.11.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Fernandez y Branas,F

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 95/01202

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANDROLOGIA, vol. 17, no. 2, 1985 BERLIN, pages 150-156, MIZUTANI T. ET AL 'Motility of seminal plasma-free spermatozoa in the presence of several physiological compounds' see the whole document	1-8
X,P	JOURNAL OF ENDOCRINOLOGY, vol. 144, February 1995 LONDON, pages 369-378, VINSON G.P. ET AL 'Type 1 angiotensin II receptors in rat and human sperm' see the whole document	1-8

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB95/01202

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
See Annex!

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB95/01202

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark: Although claims 1,3,5 (all partially and as far as in concerns "in vito" method) are directed to a method of treatment of the human/ animal body the search has been carried out and based on the alleged effects of the compound.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 95/01202

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9509186	06-04-95	AU-B- 7703294	18-04-95

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